TOXIGENIC MOULDS IN PESTICIDE-TREATED LIQUID MEDIUM

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ABSTRACT - The effect of different concentrations (10-100 µg ml^{-1}) of four pesticides (three fungicides and one insecticide) on production of three different mycotoxins in liquid medium was examined. Two toxigenic isolates producing citrinin (*Penicillium chrysogenum* and *P. corylophilum*), one producing ochratoxin A (*P. funiculosum*) and one producing zearalenone (*Fusarium moniliforme*) were selected in this study. The insecticide Actellic did not affect the mycotoxins production by tested isolates at any dose used. Ochratoxin A and zearalenone formation was completely inhibited by 50 and 100 µg ml^{-1} of each of the three fungicides used (Vitavax-Captan, Rizolex-T and Sumisclex). Rizolex-T (at 100 µg ml^{-1}) completely inhibited citrinin production by both *P. chrysogenum* and *P. corylophilum*. On the other hand, Sumisclex (at 100 µg ml^{-1} also) did not affect citrinin synthesis by *P. chrysogenum* but completely inhibited its formation by *P. corylophilum*.

RÉSUMÉ - Les effets de différentes concentrations (10-100 µg ml^{-1}) de quatre pesticides (trois fongicides et un insecticide) ont été étudiés sur la production en milieu liquide de trois mycotoxines différentes. Cette étude a été menée sur deux isolats producteur de citrinine (*Penicillium chrysogenum* et *P. corylophilum*), un isolat producteur d'ochratoxine A (*P. funiculosum*) et un isolat producteur de zéaralénone (*Fusarium moniliforme*). L'insecticide (Actellic) n'affecte pas la production de mycotoxines quelle que soit la dose utilisée. La production d'ochratoxine A et de zéaralénone est complétement inhibée par des doses de 50 µg ml^{-1} de chacun des trois fungicides étudiés (Vitavax-Captan, Rizolex-T et Sumisclex). Le Rizolex-T, à la concentration de 100 µg ml^{-1} inhibe totalement la production de citrinine par *P. chrysogenum* ou *P. corylophilum*, alors qu'à la même concentration, le Sumisclex inhibe la production de citrinine par *P. corylophilum* mais ne présente pas d'action sur la production par *P. chrysogenum*.

KEY WORDS - Citrinin, Ochratoxin A, Zearalenone, Pesticides, Toxigenic moulds.

INTRODUCTION

Mycotoxin contamination of grains and seeds is a serious economic and health hazard to both livestock and human population. The presence of these highly toxic substances in a variety of foods and feeds has led to extensive research concerning their production, detoxification and incidence. A lot of research has been done on the effect of fungistatics (natural or manufactured ones), fungicides and insecticides on fungal growth and toxigenesis, in regard to protection of foods, feeds and feedstuffs (Bell &

Doupnik, 1970; Vandergraft et al., 1973a,b, 1975; Wu & Ayres, 1974; Draughon & Ayres, 1978, 1980; Bean & Southall, 1983; Draughon, 1983; Draughon & Churchville, 1985).

In the present investigation, the effect of four pesticides on formation of each of citrinin, ochratoxin A and zearalenone by the respective toxigenic moulds in liquid culture medium was studied.

MATERIALS AND METHODS

Cultures of four toxigenic fungal species namely *Penicillium chrysogenum* Thom, *P. corylophilum* Dierckx (citrinin producers), *P. funiculosum* Thom (ochratoxin A producer) and *Fusarium moniliforme* Sheldon (zearalenone producer) isolated from pesticide-free corn grains (Hasan, 1988) were selected as test organisms. Fifty ml portions of yeast extract peptone-Czapek's supplemented medium (of the following composition:glucose, 10; peptone, 10; $NaNO_3$, 2; K_2HPO_4, 1; yeast extract, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 10.0l; g/liter of distilled water, the final pH was adjusted to 6.0 using 0.1 N HCl) were dispensed into each of 250 ml Erlenmeyer conical flasks. Four pesticides were used in this investigation viz:Vitavax-Captan (5,6-dihydro-2 methyl-1,4- oxathin-3-carboxanilido/N-(trichloromethylthio)-4-cyclohexene-1,2 dicarboximide), Rizolex T (0,0-dimethyl-0-(2,6- dichloro-4-methyl phenyl) phosphorothioate/Bis (dimethyl thiocarbomoyl) disulphide), Sumisclex (N-3,5-dichloro- phenyl)-1,2-dimethyl) cyclopropane-1, 2-dicarboximide) and Actellic (0,0-dimethyl-0-(2-dimethyl amino-6-methyl-pyrimidin ,-4-yl) phosphorothioate. Each pesticide in acetone: sterile distilled water (1:10, v/v) was added to the sterilized medium (before inoculation of the fungus) at three different concentrations 10,50 and 100 µg ml^{-1}, calculated as active ingredient. The solvent without pesticide was added to the control treatment also. One ml aliquots of the spore suspension of 1-2 week-old cultures of the test organisms were inoculated under aseptic conditions into the flasks. Four flasks were used for each treatment and control. The cultures were incubated at 28°C for 7 days as stationary cultivation. The mycelial dry weight of two flasks was determined by collecting the mycelial mats of the fungus, washed twice with distilled water, dried over night at 85°C, allowed to cool in a desiccator and then weighed. The content of each other two flasks was extracted and analysed for the respective mycotoxins of the test organisms.

Extraction of mycotoxins from fungal cultures

At the end of incubation period, the contents of each flasks (medium + mycelium) were homogenized with 100 ml of chloroform for 5 min in a high speed blender (16000 r.p.m.). For citrinin extraction, the culture medium was acidified to pH 2-3 using 6 N HCl before homogenisation. The extraction procedure was repeated 3 times. The combined chloroform extracts were washed with distilled water, dried over anhydrous sodium sulphate, filtered and concentrated under vacuum to near dryness, and diluted to 1 ml with chloroform.

Detection of mycotoxins

Chromatographic analysis of the chloroform extracts was achieved on precoated silica gel plate type 60, F 254 (Merck). The samples were applied as 0.01 ml solution using micropipettes, and the spots were dried during application with a flow of cold air. Different standard mycotoxin references (purchased from Makor Chemical Ltd. Jerusalem, Israel) were used. The potential lower limit of visual detection is ranged from 0.02-0.2 ppm. Thin layer plates were developed in both toluene: ethylacetate: formic acid (6:3:1, v/v/v) and chloroform: methanol (97:3, v/v) and treated according to the methods of Scott et al. (1970), and Gimeno (1979). Citrinin fluoresces lemon yellow under long wave UV light (Saito et al., 1971), while ochratoxin A fluoresces greenish-blue (Broce, 1970). Zearalenone was determined quantitatively according to Mirocha et al. (1974).

RESULTS AND DISCUSSION

The results in table 1 show the effects of four pesticides when incorporated into liquid medium at 10,50 and 100 µg ml^{-1} on the mycelial growth and production of citrinin, ochratoxin A and zearalenone by toxigenic moulds. Vitavax-captan inhibited the mycelial dry weight of *P. chrysogenum* (at 100 µg ml^{-1}), *P.funiculosum* (at 50 and 100 µg ml^{-1}) and *F. moniliforme* (at the three concentrations tested). The mycelial dry weight of *P. funiculosum* and *F. moniliforme* was reduced at 50 and 100 µg ml^{-1} of Rizolex-T, respectively. Sumisclex reduced the mycelial dry weight of *P. corylophilum, P. funiculosum* (using the three doses used) and *F. moniliforme* (using 100 µg ml-1). The insecticide Actellic did not affect the mycelial dry weight of any tested fungal species.

Rizolex-T proved to be the most effective pesticide tested for inhibiting citrinin production (table 1). Rizolex-T composed of two components, Rizolex (organophosphate group of fungicides) and Thiram (dithiocarbamate group). The results obtained in this investigation are in harmony with the finding of Draughon & Ayres (1978). They reported that of 13 compounds tested, the organophosphate insecticides Malathion, Diazinon, Dichlorvos and Naled proved to be the most effective for inhibiting citrinin production by *Penicillium citrinum* Nrrl 5927.

The fungicide Sumisclex proved to be the second most effective pesticide tested for preventing citrinin production. This fungicide completely inhibited citrinin production by *Penicillium corylophilum* when added to the culture medium at 50 µg ml^{-1}, but it did not affect citrinin production by the other test organism (*P. chrysogenum*). Various effects of ▪ given pesticide on production of certain mycotoxins by different strains of fungi were previously recorded. Bell & Doupnik (1970) screened some chemical used in foods or feeds as preservatives for prevention of *Aspergillus flavus* and *A. parasiticus* and aflatoxin accumulation in peanut pods. They reported that results varied from complete inhibition of fungi with no aflatoxin produced to aflatoxin found in amounts up to twice that of the control. Vandegraft et al. (1973a) also reported that insecticide treatment of wheat both increased and decreased aflatoxin and ochratoxin production depending on the fungal strains used.

		P. chrysogenum		*P. corylophilum*		*P. funiculosum*		*F. moniliforme*	
Pesticide	Concentration (µg ml⁻¹)	Dry weight	Citrinin	Dry weight	Citrinin	Dry weight	Ochratoxin	Dry weight	Zearalenone
Vitavax-Captan	0		++++		++++		++		++
	10	110	++++	129	++++	86	++++	43*	+
	50	88	++++	109	++++	1*	-	6*	-
	100	10*	++++	102	++++	0*	-	3*	-
Rizolex-T	0		++++		++++		++		++
	10	112	+++	90	++++	106	++++	70	-
	50	102	++	85	++	69	-	25*	-
	100	103	-	93	-	34*	-	11*	-
Sumisclex	0		++++		++++		++		++
	10	94	++++	74*	++	13*	-	91	++
	50	78	++++	84*	-	30*	-	90	-
	100	70	++++	23*	-	24*	-	52*	-
Actellic	0		++++		++++		++		++
	10	88	++++	106	++++	97	++	80	++
	50	84	++++	103	++++	87	++	97	++
	100	96	++++	95	++++	100	++	93	++

- = no detection of mycotoxin ; + = trace production; ++ = low production; +++= moderate production, ++++ = high production , * Means significant difference compared to the control at 5% level.

Table I: Mycelial dry weight (% of the control) and production of citrinin, ochratoxin A, and zearalenone by toxigenic moulds grown in liquid medium (28 C, 7 days) with different concentrations of pesticides.

Tableau 1: poids sec (en % par rapport au témoin) et productions de citrine, d'ochratoxine A et de zearalenone en milieu liquide (28°C) après 7 jours de culture en présence d'une game de concentration des différents pesticides.

At concentration of 50 μg ml^{-1} both Vitavax-Captan and Rizolex-T inhibited ochratoxin A production. Sumisclex proved to be the most effective pesticides tested, it prevented ochratoxin A formation when added to the culture medium at 10 μg ml^{-1} . The ability to inhibit ochratoxin A synthesis at lower concentrations was a desirable attribute because of the rapidity of deterioration of the pesticide in the environment. At the same concentration (10 μg ml^{-1}) both Vitavax-Captan and Rizolex-T stimulated production of ochratoxin A by *P. funiculosum*. Stimulation of toxin formation was previously recorded when some pesticides were used for prevention of these toxins. Draughon (1983) and Bean and Southall (1983) found that some insecticides (Dursban & Nellite) and herbicides (Pyridazinones) increased aflatoxin production by *A. flavus* and *A. parasiticus*. Draughon (1983) warned that certain insecticides now being applied to crops for insect control could stimulate growth and aflatoxin production by the toxigenic moulds.

Various insecticides and fumigants have been used , to treat stored grains in an effort to reduce contamination with mycotoxins. None of the insecticides or fumigants reduced ochratoxins formation by inoculated fungi to make their use practical (Vandegraft et al., 1973 a,b). However, Vandegraft et al. (1975) reported that both ammonia and 1% propionic acid reduced mould growth and subsequent ochratoxin formation by *A. ochraceus* Nrrl 3174. It has been established that addition of caffeine to microbiological media at levels ≥ 1 mg/ml inhibits aflatoxin and ochratoxin A production while theophylline and theobromine have little activity (Buchanan & Fletcher, 1978; Buchanan et al., 1978; 1982).

Wu & Ayres (1974) studied the effect of the organophosphate Dichlorvos on the production of ochratoxin A by different strains of *A. ochraceus* grown in liquid medium and on corn. Production of ochratoxin A was reduced by a concentration of 1 mg/100 ml of medium or g of corn and the reduction varied from 21 to 29%. When the concentration of Dichlorvos was 10 mg/100 ml or g of substrate, ochratoxin A production was reduced by 42-72% depending on fungal strain and substrate.

Production of zearalenone by *Fusarium moniliforme* was completely inhibited by the addition of Vitavax-Captan and Sumisclex at concentration of 50 μg ml^{-1}. However, Rizolex-T proved to be the most effective of the different pesticides tested. Zearalenone production was completely inhibited at 10 μg ml^{-1} of Rizolex-T. Screening for the ability of different isolates of *F. moniliforme* isolated from Egyptian cereal grains and cotton seeds indicated that about 31% and 37% of the total isolates produced zearalenone, respectively (El-Kady & El-Maraghy, 1982). The results obtained in this investigation agree with those obtained in many previous investigations. Previously reported work which screened selected pesticides for their ability to inhibit zearalenone production demonstrated that this toxin was completely inhibited by organophosphate pesticides Naled (Dibrom), Dichlorvos, EPN, Fonofos (Dyfonate), Fensulphothion (Dasanit) and Malathion (Wolf et al., 1972, Wolf & Mirocha, 1973; Rao & Harein, 1973; Berisford & Ayres, 1976a,b; Draughon & Churchville, 1985), carbamate and dithiocarbamate pesticides Metalkamate (Bux), Carbaryl (Sevin), Carbofuran (Furadan) and Maneb (Dithan M-22) (Draughon & Churchville, 1985).

Since Rizolex-T and Sumisclex have been found to inhibit not only aflatoxin formation but also citrinin, ochratoxin A and zearalenone. All of these compounds are synthesized via polyketide metabolic pathways. Rizolex-T and Sumisclex as well as

other pesticides may inhibit a key enzyme(s) in polyketide pathways and perhaps inhibit synthesis of other fungal metabolites which follow this pathway. Evidently, some characteristics of Rizolex-T and Sumisclex make them highly toxic to a diverse group of fungi.

Although the insecticide Actellic is one of the organophosphate insecticides family, however this insecticide proved to be inactive for control the production of any of the toxins under investigation (citrinin, ochratoxin A and zearalenone). Various workers have reported that organophosphate group of insecticides vary in their ability to inhibit production of aflatoxin, citrinin and zearalenone (Draughon & Ayres, 1980). The phosphate group present in the insecticide Actellic as P=S group. It has been previously reported that organophosphate fungicides have specific structural features associated with antifungal activity. Unlike insect, fungi cannot oxidize the bond P=S to P=0 and therefore, pesticides having the P=S structural feature are generally inactive against fungi unless the phosphate group is bonded to nitrogen HN-P=S (Cremyln, 1978).

REFERENCES

BEAN G.A. and SOUTHALL A., 1983 - Effect of pyridazinone herbicides on growth and aflatoxin release by *Aspergillus flavus* and *Aspergillus parasiticus*. *Appl. Environ. Microbiol.*, 146: 503-505.

BELL D.K. and DOUPNIK B.JR., 1970 - Efficacy of chemicals for control aflatoxins in peanut pods. *J. Amer. Peanut Res. Ed. Ass.* 12:35.

BERISFORD Y.C. and AYRES J.C., 1976a - Effect of insecticides on growth and zearalenone (F-2) production by the fungus, *Fusarium graminearum*. *J. Environ. Entomol.* 15:644-648.

BERISFORD Y.C. and AYRES J.C., 1976b - Use of insecticide Naled to control zearalenone production. *J. Agric. Food. Chem.* 124: 973-975.

BROCE D., 1970 - The extraction, purification, detection, quantitative measurements and confirmation of ochratoxin A,B and C of *Aspergillus ochraceus*. Abst. Int. B-30: 3059-3060.

BUCHANAN R.L. and FLETCHER A.M., 1978 - Methylxanthine inhibition of aflatoxin production. *J. Food Sci.* 43: 654-655.

BUCHANAN R.L., APPLEBAUM R.S. and CONWAY P., 1978 - Effect of theobromine on growth and aflatoxin production by *Aspergillus parasiticus*.. *J Food Safety*, 11:211-216.

BUCHANAN R.L., TRICE G. and MARINO D., 1982 - Caffeine inhibition of ochratoxin A production. *J. Food Sci.* 147: 319-321.

CREMYLN R., 1978 - Pesticides: preparation and mode of action. Wiley and Sons, New York. ,

DRAUGHON F.A., 1983 - Control or suppression of aflatoxin production with pesticides. *South. Coop. Ser. Bull.* 279: 81-86.

DRAUGHON F.A. and AYRES J.C., 1978 - Effect of selected pesticides on growth and citrinin production by *Penicillium citrinum*. *J. Food Sci.* 143: 576-578.

DRAUGHON F.A. and AYRES J.C., 1980 - Insecticide inhibition of growth and patulin production in *Penicillium expansum, Penicillium urticae, Aspergillus clavatus, Aspergillus terreus* , and *Byssochlamys nivea*. *J. Agric. Food Chem.* 128: 1115-1117.

DRAUGHON F.A. and CHURCHVILLE D.C., 1985 - Effect of pesticides on zearalenone production in culture and in corn plants. *Phytopathology* 175: 553-556.

GIMENO A., 1979 - Thin-layer chromatographic determination of aflatoxins, ochratoxins, sterigmatocystin, zearalenone, citrinin, T-2 toxin, diacetoxyscirpenol, penicillic acid, patulin and penitrem. *A.J. Assoc. Off. Anal. Chem.* 162: 579-585.

HASAN A.H., 1988 - Selective effect of some pesticides on mycoflora and mycotoxin production in corn-grains and sunflower seeds. M.Sc. Thesis, Botany Depart. Fac. of Sci., Assiut Univ., Assiut, Egypt.

MIROCHA C.J., SCHAUERHAMER B. and PATHRE S.V., 1974 - Isolation, detection and quantitation of zearalenone in maize and barley. *J. Assoc. Off. Anal. Chem.* 157: 1104-1110.

RAO H.R.G. and HAREIN P.K., 1973 - Inhibition of aflatoxin and zearalenone biosynthesis with Dichlorvos. *Bull. of Environm. Contam. Toxicol.*, 10: 112-115.

SAITO M., ENOMOTO M. and TATSUNO T., 1971 - Yellowed rice toxins, Luteoskyrin and related compounds, chlorine containing compounds and citrinin. *In:* A. Ciegler, S. Kadis and S.J. Ajl (Eds) Microbial Toxins Vol VI Fungal toxins. Academic Press, New York and London.

SCOTT P.M., LAWRENCE J.W. and VAN WALBEAK W., 1970 - Detection of mycotoxins by thin-layer chromatography. Application to screening of fungal extracts. *Appl. Microbiol.* 120: 839-842.

VANDEGRAFT E.E., SHOTWELL O.L., SMITH M.L. and HESSELTINE C.W., 1973a - Mycotoxins production affected by insecticide treatment of wheat. *Cereal Chem.* 50: 264-270.

VANDEGRAFT E.E., SHOTWELL O.L., SMITH M.L. and HESSELTINE C.W., 1973b - Mycotoxin formation affected by fumigation of wheat. *Cereal Sci. Today* 118:412.

VANDEGRAFT E.E., HESSELTINE C.W. and SHOTWELL O.L., 1975 - Grain Preservatives: Effect on aflatoxin and ochratoxin production. *Cereal Chem.* 152: 79-84.

WOLF J.C. and MIROCHA C.J., 1973 - Cited in Berisford, Y.C. and Ayres, J.C. 1976: use of the Naled to control zearalenone production. *J. Agric. Food Chem.* 124: 973-957.

WOLF J.C.,LIEBERMAN J.R. and MIROCHA C.J., 1972 - Inhibition of F-2 (Zearalenone) biosynthesis and perithecium production in *Fusarium roseum* "Graminearum" *Phytopathology* 162: 937-939.

WU M.T. and AYRES J.C., 1974 - Effect of Dichlorvos on ochratoxin production by *Aspergillus ochraceus*. *J. Agric. Food Chem.* 122: 536-537.